8-Isoprostane EIA Kit

Catalog No. 516351

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CONTENTS OF THE KIT

Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
1	8-Isoprostane EIA Antiserum	1 vial/100 dtn	1 vial/500 dtn
2	8-Isoprostane AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
3	8-Isoprostane EIA Standard	1 vial/1 each	1 vial/1 each
4	EIA Buffer Concentrate	2 vials/10 ml	4 vials/10 ml
5	Wash Buffer Concentrate	1 vial/5 ml	1 vial/12.5 ml
5a	Tween 20	1 vial/3 ml	1 vial/3 ml
6	Mouse Anti-rabbit IgG Coated Plate	1 plate/1 each	5 plates/1 each
7	Plate Cover	1 cover/1 each	5 covers/1 each
8	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

PRECAUTIONS

- Please read these instructions carefully before beginning this assay.
- The reagents in this kit have been tested and formulated to work exclusively with ACE™ EIA kits. This kit may not perform as described if any reagent or procedure is replaced or modified.
- For research use only. Not for human or diagnostic use.

WARRANTY AND LIMITATION OF REMEDY

Cayman Chemical Company makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery. Buyer's exclusive remedy and Cayman Chemical Company's sole liability hereunder shall be limited to refund of the purchase price of, or at Cayman Chemical Company's option, the replacement of, all material that does not meet our specifications. Cayman Chemical Company shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling. Said refund or replacement is conditioned on Buyer giving written notice to Cayman Chemical Company within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within said thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

IF YOU HAVE PROBLEMS

Our technical support staff may be reached by phone (800-364-9897, 734-971-3335), fax (734-971-3640), or E-Mail (techserv@caymanchem.com) Monday through Friday 8:00 AM to 6:00 PM EST. In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Cayman Chemical offers an introductory course in EIA theory and practice. Please contact our Customer Service Department for more information.

STORAGE AND STABILITY

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

ADDITIONAL ITEMS REQUIRED

1. A plate reader with a 405-420 nm filter.
2. An adjustable pipettor.
3. A source of "UltraPure" water. Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants ("UltraPure"). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA. [NOTE: Ultrapure water is available for purchase (Catalog No. 400000).]
4. Materials used for sample preparation (see page 6).

ABOUT THIS ASSAY

Biochemistry of 8-Isoprostane

The isoprostanes are a family of eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals. Isoprostanes appear as artifacts in tissue and plasma samples which have undergone oxidative degradation during prolonged or improper storage. They also appear in the plasma and urine under normal conditions and are elevated by oxidative stress.

At least one of the isoprostanes, 8-isoprostane (8-*epi* $PGF_{2\alpha}$), has been shown to have biological activity. It is a potent pulmonary and renal vasoconstrictor[1] and has been implicated as a causative mediator of hepatorenal syndrome and pulmonary oxygen toxicity.[2] 8-Isoprostane has been proposed as a marker of antioxidant deficiency and oxidative stress and elevated levels have been found in heavy smokers.[3] 8-Isoprostane levels are also a relative indicator of sample integrity for lipid-containing samples such as serum, plasma, and whole cell preparations.[4] Plasma from healthy volunteers contains modest amounts of 8-isoprostane (40-100 pg/ml) that increase with the age of the test subject.[5] Normal human urinary levels range from 10-50 ng/mmol creatinine, which is an order of magnitude higher than many enzymatically derived eicosanoids.[5,6] A scheme of 8-isoprostane generation is shown in Figure 1 below.

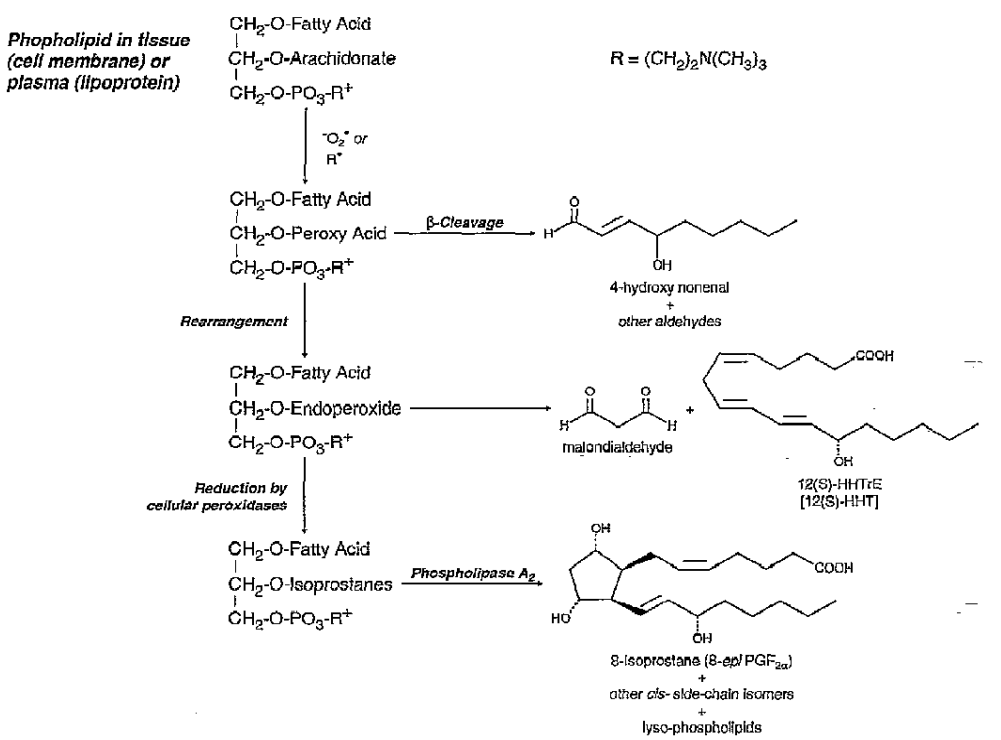


Figure 1. The formation of 8-isoprostane

INTRODUCTION TO ACE™ EIA s

Description of the ACE™ Competitive Enzyme Immunoassay[7,8]

This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. Because the concentration of the 8-isoprostane tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-isoprostane tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. This rabbit antiserum-8-isoprostane (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound 8-Isoprostane Tracer}] \propto 1/[\text{8-Isoprostane}]$$

A schematic of this process is shown in Figure 2.

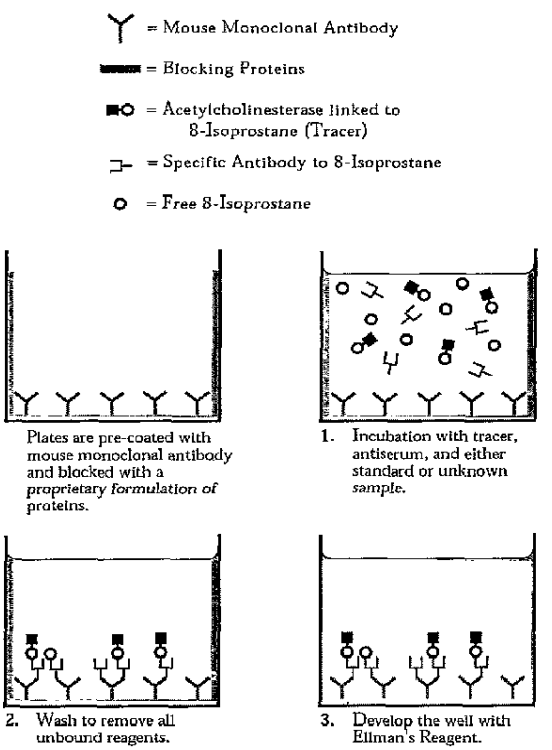


Figure 2. Schematic of the ACE™ EIA

Biochemistry of ACE™ EIAs

The electric organ of the electric eel, *Electrophorus electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in ACE™ enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 3). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows multiple development of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. Since AChE is stable during the development step, it is unnecessary to use a "stop" reagent, and the plate may be read whenever it is convenient.

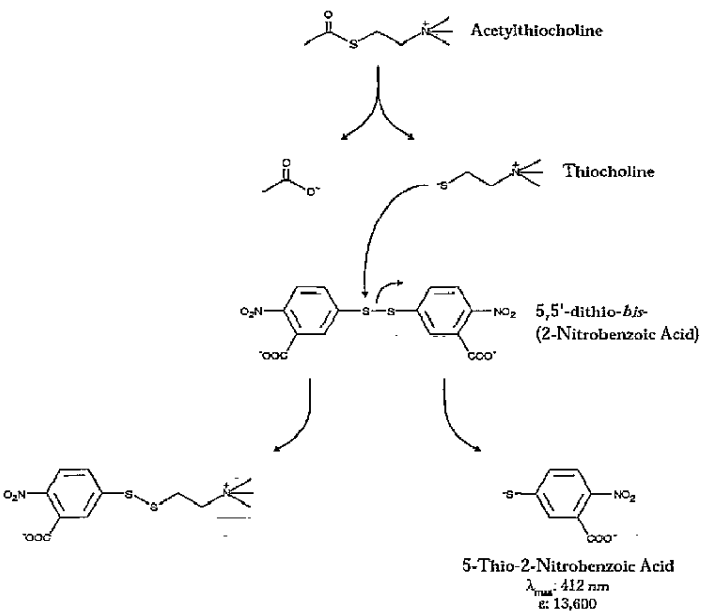


Figure 3. Reaction catalyzed by AChE

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. Even freshly prepared Ellman's Reagent has some measurable absorbance, approximately 0.1 Absorbance Units (A.U.). The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antiserum a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antiserum can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values versus concentration of a series of wells containing various known amounts of analyte.

PRE-ASSAY PREPARATION

Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants ("UltraPur e"). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water , and sterile water (for injections) are not adequate for EIA. (Catalog No. 400000)

Buffer Preparation *(Store all buffers at 4°C)*

1. EIA Buffer Preparation

   Dilute the contents of one vial of EIA Buffer Concentrate (vial #4) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. (NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.)

2. Wash Buffer Preparation

   Dilute the contents of the vial (5 ml) of Wash Buffer Concentrate (vial #5) to a total volume of 2 liters with UltraPure water and add 1 ml of Tween 20 (vial #5a), or dilute the contents of the vial (12.5 ml) of Wash Buffer Concentrate (vial #5) to a total volume of 5 liters with UltraPure water and add 2.5 ml of Tween 20 (vial #5a). (NOTE: Tween 20 is a viscous liquid and cannot be measured by a pipet. A positive displacement device such as a syringe should be used to deliver small quantities accurately.) A smaller volume of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Tween 20 (0.5 ml/liter of Wash Buffer).

Sample Preparation

This assay has been validated for a wide range of samples including urine and plasma. Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

In general, tissue culture supernatant samples may be diluted with EIA buffer and added directly to the assay well. Plasma, serum, urine, whole blood, as well as other heterogeneous mixtures such as lavage fluids and aspirates often contain contaminants which can interfere in the assay. It is best to check for interference before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between 10 and 100 pg/ml. If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated 8-isoprostane concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. The Purification Protocol below is one such method.

Cayman Chemical Company offers an 8-Isoprostane Affinity Column and Affinity Sorbent (Catalog Nos. 416358 and 416359) for simpler purification of samples. The affinity column purification procedures were validated with plasma and urine samples. Recoveries averaged >90% with a variance of <20%. The SPE (solid phase extraction) purification methods described (see page 9) were validated by a comparison of the data from EIA and gas chromatography/negative ion chemical ionization-mass spectrometry (GC/NICI-MS). GC/NICI-MS analysis was performed on samples derivatized as pentafluorobenzyl esters and *tert*-butyldimethyl-silyl ethers.[9]

General Precautions

1. All samples must be free of organic solvents prior to assay.
2. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C in the presence of 0.005% BHT. Storage at -20°C is insufficient to prevent oxidative formation of 8-isoprostane.[9]

1. Lavage Fluids and Aspirates

   Some lavage fluids may be assayed without any purification. Be certain to dilute the standards in the same medium as your samples. [NOTE: If you obtain inconsistent results, SPE or immunoaffinity purification is warranted.]

2. Urine

   Urine samples give excellent correlation to GC/MS if purified by SPE and TLC (see Figure 4, page 7) or immunoaffinity methods prior to analysis.

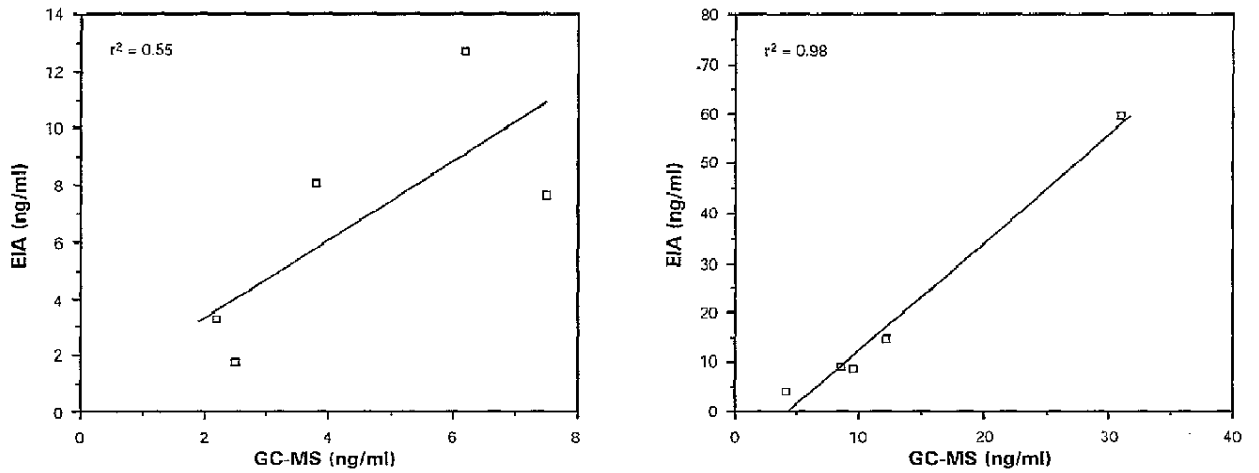


Figure 4. Comparison of urine samples purified by SPE (left) and by SPE and TLC (right).

3. Culture Media

Most culture media samples can be assayed without purification. If the estimated concentration of your samples is too low to allow dilution with EIA Buffer, be certain to dilute the EIA standards in the same medium as your samples. [NOTE: If you obtain inconsistent results, SPE or immunoaffinity purification is warranted.]

4. Plasma

Less than half of total plasma 8-isoprostane is present as the free acid, while the remainder is esterified in lipoproteins.[3] Direct EIA of plasma samples without hydrolysis will measure only the free 8-isoprostane fraction. Total plasma 8-isoprostane determination requires an alkaline hydrolysis prior to EIA (see page 8).

Plasma samples give excellent correlation to GC/MS if purified prior to analysis (see Figure 5). Analysis of plasma samples without purification may lead to inconsistent results. If inconsistent results are obtained, we recommend the immunoaffinity purification as the easiest and most convenient purification format (see Figure 6, page 8).

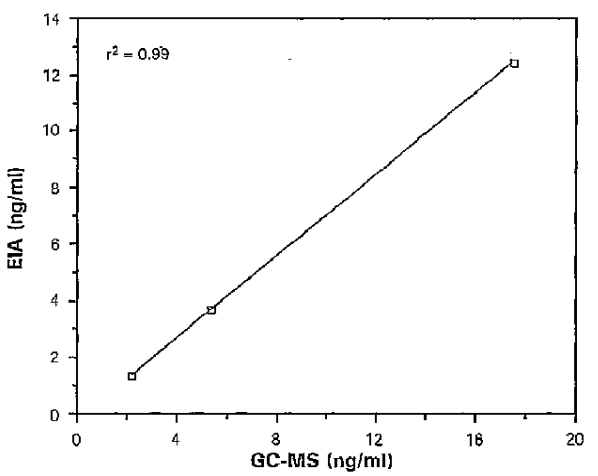


Figure 5. Correlation of plasma samples purified by SPE.

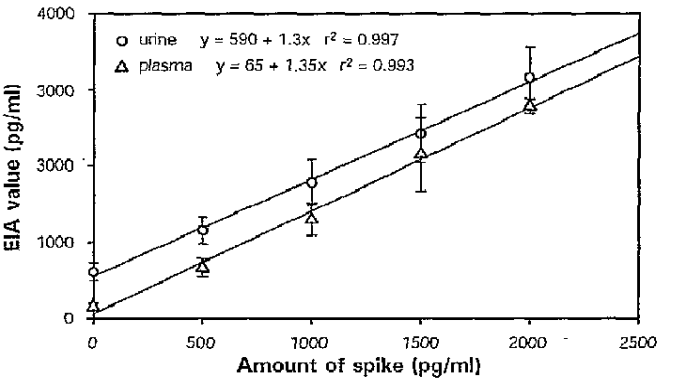


Figure 6. Correlation of plasma and urine samples from immunoaffinity purification.

5. Tissue

Tissue samples should be homogenized using a polytron type homogenizer and then processed as plasma samples. Again, most of the 8-isoprostane will be esterified in lipids, so hydrolysis must be performed in order to determine total amounts of 8-isoprostane.

SAMPLE PURIFICATION

All purifications can be checked for recovery by adding a radioactive tracer at the appropriate step. Tritiated $PGF_{2\alpha}$ can be used as a surrogate for tritiated 8-*iso* $PGF_{2\alpha}$ in the SPE purification if the later is unavailable.

A. Determination of Free 8-Isoprostane

1. Preparation for Affinity Sorbent/Column Purification

Urine samples should be centrifuged briefly to remove sediment. Centrifuge plasma samples briefly and dilute 1:5 in Eicosanoid Affinity Column Buffer (0.1 M potassium phosphate, pH 7.4, containing 0.5 M NaCl and 0.05% NaN_3; this buffer may also be purchased as Catalog No. 400220). Proceed with the purification using the 8-Isoprostane Affinity Sorbent, Column, or Purification Kit (Catalog Nos. 416358, 416359, and 516358).

2. Preparation for SPE Purification

a. Aliquot a known amount of sample (1 ml plasma or 1 g homogenized tissue will be used as an example) to a test tube. Add 10,000 cpm of tritium-labeled 8-*iso* $PGF_{2\alpha}$ ([^{3}H]-8-*iso* $PGF_{2\alpha}$ (Catalog No. 216350)) or [^{3}H]-$PGF_{2\alpha}$.

b. Add 2 ml ethanol to the sample and vortex. Allow the samples to stand at 4°C for 5 minutes, then centrifuge at 1,500 x g for 10 minutes to remove precipitated proteins. (NOTE: Precipitation of proteins using ethanol is optional and may not be needed if samples are clean enough to flow through the SPE.)

c. Decant the supernatant into a clean test tube.

d. Evaporate the ethanol either by vacuum centrifugation or under a gentle stream of nitrogen.

e. Adjust the pH of the sample to ~4.0 using 1.0 M acetate buffer or citrate buffer (pH 4.0). (Standardize the pH adjustment using the sample matrix prior to proceeding with a large number of samples; approximately 1-2 equivalents of buffer is required for most biological samples.) If the samples are cloudy or contain precipitate, either filter or centrifuge to remove the precipitate. Particulate matter in the sample may clog the SPE cartridge, resulting in loss of the sample.

f. Proceed to the Purification Protocol for the SPE cartridge (see page 9).

B. Determination of Total (free and esterified) 8-Isoprostane

1. Aliquot a known amount of sample (1 ml plasma or 1 g homogenized tissue will be used as an example) to a test tube. If SPE purification will be utilized, add 10,000 cpm of [^{3}H]-8-*iso* $PGF_{2\alpha}$ or [^{3}H]-$PGF_{2\alpha}$.

2. Add an equal volume of 15% wt/vol KOH and incubate at 40°C for 60 minutes.

3. Add 2 ml ethanol containing 0.01% BHT to the sample and vortex. Incubate at 4°C for 5 minutes, then centrifuge at 1,500 for 10 minutes to remove precipitated proteins. [NOTE: Precipitation of proteins using ethanol is optional and may not be needed if samples are clean enough to flow through the SPE.]
4. Decant the supernatant into a clean test tube.
5. Evaporate the ethanol either by vacuum centrifugation or by evaporation under a gentle stream of nitrogen.
6. Continue to process the samples as described below for the appropriate technique being utilized.

   a. Affinity Sorbent/Column Purification

      After the ethanol has been evaporated, neutralize the sample to ~pH 7.0-7.4 by the addition of 1 M KH_2PO_4 (~3 ml). (Standardize the pH adjustment using the sample matrix prior to proceeding with a large number of samples; approximately 1-2 equivalents of buffer is required for most biological samples.) Add 1-2 ml of Eicosanoid Affinity Column Buffer (0.1 M potassium phosphate, pH 7.4, containing 0.5 M NaCl; this buffer may also be purchased as Catalog No. 400220). Proceed with the purification using the 8-Isoprostane Affinity Sorbent, Column, or Purification Kit (Catalog Nos. 416358, 416359, and 516358).

   OR

   b. SPE/TLC Purification

      After the ethanol has been evaporated, acidify the sample to ~pH 4.0 by the addition of 30% acetic acid (~2-2.5 ml) or 1.0 M acetate buffer or citrate buffer (pH 4.0). (Standardize the pH adjustment using the sample matrix prior to proceeding with a large number of sample.) Proceed with the purification as described below.

Purification Protocol (SPE/TLC method)

A. Activate a C-18 SPE cartridge (Catalog No. 400020) by rinsing with 5 ml methanol and then with 5 ml UltraPure water. Do not allow the cartridge to become dry.

B. Pass the sample [either from steps A2f or B6b, or from urine samples, which have been adjusted to pH ~4.0 using 1.0 M acetate buffer or citrate buffer (pH 4.0) and spiked with 10,000 cpm of [^{3}H]-8-*iso* $PGF_{2\alpha}$ (Catalog No. 216350) or [^{3}H]-$PGF_{2\alpha}$, through the SPE cartridge. Rinse the cartridge with 5 ml UltraPure water (allow the cartridge to become dry after this step), followed by 5 ml HPLC grade hexane. Discard both the washes. Elute the 8-isoprostane with 5 ml ethyl acetate containing 1% methanol.

C. Remove 10% of the eluate for scintillation counting.*

D. Evaporate the ethyl acetate to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen. For plasma, serum, and most lavage fluids, skip to step H.

E. Dissolve the sample in a small amount of acetone and spot in the preadsorbent zone of a channeled 20 x 20 cm TLC plate without any fluorescent indicator (e.g., Analtech 31911, Whatman 4865-821). The preadsorbent zone of the plates will concentrate the sample into a thin line at the solvent front so there is no need for special precautions when spotting the sample. At least 1 µg of authentic 8-isoprostane (Catalog No. 16350) must be spotted on one of the edge lanes of each plate to help locate the appropriate bands in your sample. Develop the plate using chloroform/methanol/acetic acid/water (80:18:1:0.8, v/v).

F. After the solvent has traveled to the top of the plate, remove the plate from the solvent chamber and allow to dry. The band containing 8-isoprostane may be detected in one of two ways: masking all of the lanes except the one containing the authentic standard and spraying this lane with 3.5% phosphomolybdic acid, or by carefully spreading a mixture of iodine and silica gel on the 8-isoprostane lane and removing after 5 minutes.

G. Carefully scrape the band corresponding to 8-isoprostane from each sample lane onto a piece of weighing paper and transfer into a clean test tube. Elute the sample by adding 4 ml ethanol, vortexing, and then centrifuging at 1,500 x g for 10 minutes. Decant the supernatant into a clean test tube and evaporate the solvent using either a vacuum centrifuge or a stream of dry nitrogen.

H. Add 450 µl of EIA Buffer and vortex. Use this for EIA analysis. It is common for an insoluble precipitate to remain after the addition of EIA buffer; this will not affect the assay.

*If it is necessary to stop during this purification, samples may be stored in the ethyl acetate/methanol solution at -20°C or -80°C.

Preparation of Assay-specific Reagents

1. 8-Isoprostane Standard

   Equilibrate a pipet tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipet tip, transfer 100 µl of the 8-Isoprostane Standard (vial #3) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 5 ng/ml. Store this solution at 4°C; it will be stable for approximately six weeks.

   (NOTE: If assaying culture media samples that have not been diluted with EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve).

   To prepare the standard for use in EIA: obtain 8 clean test tubes and number them #1 through #8. Aliquot 900 µl EIA Buffer to tube #1 and 500 µl EIA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (5 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

2. 8-Isoprostane AChE Tracer

   Reconstitute the 100 dtn 8-Isoprostane Tracer (vial #2) with 6 ml EIA Buffer or the 500 dtn 8-Isoprostane Tracer (vial #2) with 30 ml EIA Buffer. Store the reconstituted 8-Isoprostane Tracer at 4°C (do not freeze!) and use within four weeks. A 20% surplus of 8-Isoprostane Tracer has been included to account for any incidental losses.

3. 8-Isoprostane Antiserum

   Reconstitute the 100 dtn 8-Isoprostane Antiserum (vial #1) with 6 ml EIA Buffer or the 500 dtn 8-Isoprostane Antiserum (vial #1) with 30 ml EIA Buffer. Store the reconstituted 8-Isoprostane Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of 8-Isoprostane Antiserum has been included to account for any incidental losses.

PERFORMING THE ASSAY

Plate Set Up

The 96 well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. (NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 2-4°C. Be sure the packet is sealed with the desiccant inside.)

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate. *(NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.)* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate.

Two suggested plate formats are shown in Figure 7. The user may vary the location and type of wells present as necessary for each particular experiment. We suggest you record the contents of each well on the template sheet provided (see page 15).

Full Plate Format

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	TA	S1	S1	★	★	★	★	★	★	★	★
B	Blk	TA	S2	S2	★	★	★	★	★	★	★	★
C	Blk	NSB	S3	S3	★	★	★	★	★	★	★	★
D	Blk	NSB	S4	S4	★	★	★	★	★	★	★	★
E	Blk	B_0	S5	S5	★	★	★	★	★	★	★	★
F	Blk	B_0	S6	S6	★	★	★	★	★	★	★	★
G	Blk	B_0	S7	S7	★	★	★	★	★	★	★	★
H	Blk	B_0	S8	S8	★	★	★	★	★	★	★	★

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
★ - Sample

Partial Plate Format

	1	2	3	4
A	Blk	S1	S1	★
B	Blk	S2	S2	★
C	NSB	S3	S3	★
D	NSB	S4	S4	★
E	B_0	S5	S5	★
F	B_0	S6	S6	★
G	B_0	S7	S7	★
H	TA	S8	S8	★

Figure 7. Sample plate formats

Pipet the Reagents

PIPETTING HINTS

- **Use different tips to pipet the buffer, standard, sample, tracer, and antibody.**
- **Before pipetting each reagent, equilibrate the pipet tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).**
- **Do not expose the pipet tip to the reagent(s) already in the well.**

1. EIA Buffer

   Add 100 µl EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl EIA Buffer to Maximum Binding (B_0) wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for EIA buffer in the NSB and B_0 wells (i.e., add 50 µl culture medium to NSB and B_0 wells and 50 µl EIA Buffer to NSB wells).

2. 8-Isoprostane Standard

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipet tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipet tip in that standard.

3. Samples

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate.

4. 8-Isoprostane AChE Tracer

   Add 50 µl to each well *except* the Total Activity (TA) and the Blank (Blk) wells.

5. 8-Isoprostane Antiserum

   Add 50 µl to each well *except* the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

Incubate the Plate

Cover each plate with plastic film (item #7) and incubate for 18 hours at room temperature.

Develop the Plate

When ready to develop the plate(s), reconstitute one 100 dtn vial of Ellman's Reagent (vial #8) with 20 ml of UltraPure water, or reconstitute one 250 dtn vial of Ellman's Reagent (vial #8) with 50 ml of UltraPure water (20 ml of reagent is sufficient to develop 100 wells). Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays be run on different days.

Empty the wells and rinse five times with Wash Buffer. Add 200 µl of Ellman's Reagent to each well and 5 µl of tracer to the Total Activity wells. Cover the plate with plastic film.

Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (i.e., B_0 wells equal 0.2 A.U. (blank subtracted)) in 60-90 minutes.

Read the Plate

Read the plate(s) at a wavelength between 405 and 420 nm. Before reading each plate, wipe the bottom of the plate with a clean tissue to remove finger prints, dirt, etc., as smudges on the bottom of the plate can significantly alter absorbance readings. Be certain that the Ellman's Reagent has not splashed up on the plate cover as any loss of Ellman's Reagent will affect the absorbance readings. If it did, use a pipet to remove the Ellman's Reagent from the cover and place into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate 3 times with Wash Buffer and repeat the development with fresh Ellman's Reagent.

The plate may be checked periodically until the B_0 wells have reached a minimum of 0.2 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells is in the range of 0.3-0.8 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

CALCULATING THE RESULTS

It is usually more convenient to calculate the assay results by computer; most plate readers come with data reduction software, or a spreadsheet program can be used (4-parameter or log-logit curve fit). If the results need to be calculated manually, the procedure is as follows:

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the $\%B/B_0$ (% Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Multiply by 100 to obtain $\%B/B_0$. Repeat for S2-S8 and all sample wells.
5. The total activity values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B_0 divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the sample data (see page 13). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 14 for Troubleshooting).

Plotting the Standard Curve

Plot $\%B/B_0$ for standards S1-S8 versus 8-isoprostane concentration (usually in pg/ml) on semi-log paper.

Determining the Concentration of your Samples

Calculate the $\%B/B_0$ value for each sample. Determine the concentration of each sample by identifying the $\%B/B_0$ on the standard curve and reading the corresponding values on the x-axis. (NOTE: Remember to account for any dilutions of the sample prior to the addition to the well.) $\%B/B_0$ values greater than 80% and less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample would indicate interference which could be eliminated by purification.

Calculations

$$\text{Recovery Factor} = \frac{10 \times \text{cpm of sample}}{[^3\text{H}]\text{-8-}iso\,\text{PGF}_{2\alpha} \text{ added to sample (cpm)}}$$

$$\text{8-Isoprostane (pg) in purified sample} = \left[\frac{\text{Value from EIA (pg/ml)}}{\text{Recovery Factor}}\right] \times 0.5 \text{ ml}^* - \text{added } [^3\text{H}]\text{-8-}iso\,\text{PGF}_{2\alpha} \text{ (pg)}$$

$$\text{Total 8-Isoprostane in sample (pg/ml)} = \frac{\text{8-Isoprostane (pg) in purified sample}}{\text{Volume of sample used for purification (ml)}}$$

*Volume of reconstituted sample after purification; adjust this number accordingly if a different volume of EIA buffer was used to reconstitute the sample after purification.

PERFORMANCE CHARACTERISTICS

Precision

The intra- and interassay CV's have been determined at multiple points on the standard curve. These data are summarized in the graph below.

Specificity

8-Isoprostane	100%	2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	0.09%
8-*iso* Prostaglandin $F_{3\alpha}$	20.55%	8-*iso* Prostaglandin $F_{1\beta}$	0.08%
8-*iso* Prostaglandin E_2	1.84%	Thromboxane B_2	0.08%
2,3-dinor-8-*iso* Prostaglandin $F_{1\alpha}$	1.7%	11-dehydro Thromboxane B_2	0.07%
8-*iso* Prostaglandin E_1	1.56%	11β-Prostaglandin $F_{2\alpha}$	0.03%
Prostaglandin $F_{1\alpha}$	0.71%	Prostaglandin E_2	0.02%
Prostaglandin $F_{3\alpha}$	0.66%	8-*iso*-15(R)-Prostaglandin $F_{2\alpha}$	0.02%
Prostaglandin E_1	0.39%	13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$	<0.01%
Prostaglandin D_2	0.16%	Tetranor PGEM	<0.01%
6-keto Prostaglandin $F_{1\alpha}$	0.14%	Tetranor PGFM	<0.01%
Prostaglandin $F_{2\alpha}$	0.14%		

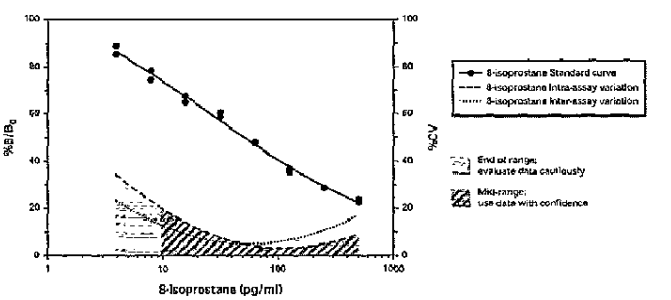
Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use this one to determine the values of your samples. Depending on the development conditions and the purity of the water used, your results could differ substantially from the data presented below. *(NOTE: Raw data is reported in milli-Absorbance Units.)*

	Raw Data		Average	Corrected
Total Activity	266	277		
NSB	3	2	2.5	
B_0	780	783	783.5	781
	786	785		

Standard Curve

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
500	185	177	182.5	174.5	23.4	22.3
250	226	224	223.5	221.5	28.6	28.7
125	289	279	286.5	276.5	36.7	35.4
62.5	376	374	373.5	371.5	47.8	47.6
31.3	475	460	472.5	457.5	60.5	58.6
15.6	529	509	526.5	506.5	67.4	64.9
7.8	585	614	582.5	611.5	74.6	78.3
3.9	669	694	666.5	691.5	85.3	88.8



50% B/B_0 - 54 pg/ml
Detection Limit (80% B/B_0) - 5 pg/ml

TROUBLESHOOTING

Problem: Erratic values; dispersion of duplicates.
Causes: Trace organic contaminants in the water source; replace activated carbon filter or change source of UltraPure water. *-or-* Poor pipetting/technique.

Problem: High NSB (>0.035).
Causes: Poor washing. *-or-* Exposure of NSB wells to specific antibody.

Problem: Very Low B_0.
Causes: Contamination of water with organic solvents. *-or-* Plate requires additional development time. *-or-* Dilution error in preparing reagents.

Problem: Low Sensitivity (shift in dose response curve).
Cause: Standard is degraded.

Problem: Analyses of two dilutions of a biological sample do not agree (i.e., more than 20% difference).
Cause: Interfering substances are present. Sample must be purified prior to analysis by EIA.[10]

Problem: Only Total Activity (TA) wells develop.
Cause: Trace organic contaminants in the water source; replace activated carbon filter or change source of UltraPure water.

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ADDITIONAL READING

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RELATED PRODUCTS

8-*iso* Prostaglandin $F_{2\alpha}$ - Cat. No. 16350 • [^{3}H]-8-*iso* Prostaglandin $F_{2\alpha}$ - Cat. No. 216350 • UltraPure Water - Cat. No. 400000 • SPE Cartridges - Cat. No. 400020 • 8-Isoprostane Affinity Column - Cat. No. 416358 • 8-Isoprostane Affinity Sorbent - Cat. No. 416359 • 8-Isoprostane EIA Kit (Solid Plate) - Cat. No. 516351.1 • 8-Isoprostane Affinity Purification Kit - Cat. No. 516358

PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES